REMARKS

Reconsideration of this application is respectfully requested. Applicants have canceled claims 89-151. Similar claims are currently pending in U.S. application No. 11/068,903.

Double Patenting Rejections

Claims 152-171 were rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-16 of U.S. Patent No. 6,525,243; claims 1-21 of U.S. Patent No. 6,147,276; and claims 1-18 of U.S. Patent No. 6,252,133 B1. Solely to expedite allowance of the pending claims, and not in acquiescence to this rejection, applicants will submit Terminal Disclaimers in compliance with 37 C.F.R. § 1.321(c) when the claims are otherwise indicated to be allowable.

Rejections under 35 U.S.C. § 101

Claims 152-171 were provisionally rejected under 35 U.S.C. § 101 as allegedly claiming the same invention as that of claims 146-163 in copending Application No. 09/225,233. The Office alleges that there is no distinction between the claimed mammals in the two applications.

Applicants traverse the rejection. Claims 146-163 in copending Application No. 09/225,233 recite that the pre-existing mammal is a "non-foetal" mammal. Claims 152-171 in the instant application do not contain this limitation. Accordingly, applicants are not claiming the same invention as that of claims 146-163 of copending Application No. 09/225,233, and respectfully request withdrawal of the rejection.

Claims 152-171 were rejected under 35 U.S.C. § 101 as allegedly being directed to non-statutory subject matter. The Office contends that the pending claims do not sufficiently distinguish over pre-existing cattle, sheep, pigs, goats, mice, and rabbits. The Office contends that the claimed subject matter is non-statutory because it is a copy or replica of a pre-existing mammal.

Applicants traverse the rejection. As the Office recognizes, the claimed subject matter is directed to a **copy or replica** of a pre-existing mammal, i.e., a clone, and not to the pre-existing mammal. A clone of a pre-existing, non-embryonic, donor mammal is never found in nature because the clone is produced asexually. Nature does not make copies, or replicas, of pre-existing mammals. The Office does not contest this point. Consequently, the "hand-of-man" is required for applicants' clone to exist, i.e., applicants' claimed subject mater is directed to a **non-naturally occurring animal** which, according to the Supreme Court, is patentable subject matter under 35 U.S.C. § 101. See, *Diamond v. Chakrabarty,* 206 U.S.P.Q. 193, 197 (1980).

Further, although applicants' clone is a copy, or replica, of a previously known mammal, it is not the same mammal because, among other things, it occupies a different space and time than the previously known mammal. In addition, the two mammals will have phenotypic and behavioral differences. (February 26, 2003, Declaration of Dr. David Wells at ¶23.) Thus, applicants' clone is distinguishable from the previously known animal.

Moreover, the Office's argument that "there is no discernible difference that lend a patentable distinction between the clone and the pre-existing mammal" is not based on any tenet of patent law. Rather, the Office's recognition that "the methods of

producing them are different" supports that the "hand of man" is required. Accordingly, applicants respectfully request withdrawal of the rejection.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 152-171 were rejected under 35 U.S.C. § 112, first paragraph, because the specification, while being enabling for clones of pre-existing cattle, sheep, pigs, and goats, allegedly does not reasonably provide enablement for clones of pre-existing mice, rabbits, horses, and rats. The Office contends that, at the time of filing, the skilled artisan would have regarded the cloning of mice, rabbits, horses, and rats to be unpredictable because each uses method steps not taught by the present specification.

Applicants traverse the rejection. At the outset, any conclusions regarding the cloning of mice, rabbits, horses, and rats must take into account the low efficiency of the cloning process. In view of the known inefficiency of the cloning process, the fact that applicants' cloning process may have to be repeated many times to assure success in mice, rabbits, horses, and rats does not mean that undue experimentation would be required. Rather, since any experimentation would be repetitive, it would be routine, and routine experimentation does not negate enablement. *See In re Wands*, 858 F.2d 731, 737; 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988). The fact that there may be ways to improve this efficiency through the use of additional steps does not negate that applicants' method is enabled. Accordingly, the Office's conclusion that cloning in mice, rabbits, horses, and rats is not enabled is in error.

Moreover, the Office has presented **no evidence** that one of skill in the art would not succeed in using applicants' claimed method to produce mice, rabbits, horses, and rats when the inefficiency of applicants' claimed process is taken into consideration. In fact, the Office has not even alleged that applicants' claimed process will not work if

nuclear transfer is performed a sufficient number of times. As a result, the Office has not fulfilled its burden of establishing a reasonable basis to question the enablement provided for applicants' claimed invention. *See In re Wright*, 999 F.2d 1557, 1562, 27 U.S.P.Q.2d 1410, 1513 (Fed. Cir. 1993).

In addition, the Office's contention that the specific cloning of mice, rabbits, horses, and rats is unpredictable because each uses method steps not taught by the present specification is in error. For each of these species, applicants' teachings, either alone or in combination with well-known techniques, enable one of skill in the art to successfully clone each referenced species. The fact that later-developed improvements in protocols improved the efficiencies as to each of these species does not render applicants' specification non-enabling as to these species.

Mice

First, as the Office points out, successful cloning of mice, as reported by Wakayama, used a "prolonged interval between nuclear injection and oocyte activation." Applicants' specification teaches such a prolonged interval between nuclear injection and oocyte activation on pages 12-13, bridging paragraph. Thus, the successful cloning of mice was performed according to applicants' teachings.

Rabbits

Second, while the cloning of rabbits, as reported by Chesne, used "asynchronous" embryo transfer, the use of asynchronous transfer with *in vitro* manipulated rabbit embryos was well-known prior to applicants' filing date. (Landa, 1981, Exhibit 1, and Al-Hasani et al., 1986, Exhibit 2.)

Landa used synchronous and asynchronous embryo transfer with *in vitro* cultured rabbit embryos, which had been previously frozen. (Landa at 265, Abstract.) Synchronous embryo transfer with *in vitro* cultured rabbit embryos, which had been previously frozen, was not considered successful since only 1 of 94 of the embryos implanted. (*Id.* at 269, paragraph 1.) In contrast, asynchronous embryo transfer with *in vitro* cultured rabbit embryos, which had been previously frozen, was more successful since 19 of 50 of the embryos implanted. (*Id.* at 269, paragraph 2.)

Al-Hasani et al. compared synchronous and asynchronous embryo transfer with *in vitro* cultured rabbit embryos. (Al-Hasani et al. at 187, Summary.) Al-Hasani et al. concluded that *in vitro* culture conditions lead to a delay in the development of embryos and that the longer the embryos are cultured *in vitro*, the greater the delay in the development of embryos. (*Id.* at 194, paragraph 1.) Al-Hasani et al. concluded that this delay in the development of embryos can be compensated, in part, by using an asynchronous recipient animal and provided optimal asynchronicities for embryos cultured *in vitro* for various periods of time. Asynchronicities in the recipients of minus 6 hours, minus 24 hours, and minus 36 hours are given as optimal for embryos cultured for different periods of time *in vitro*. (Al-Hasani et al. (1986) at 194, paragraph 1.)

Based on Landa (1981) and Al-Hasani et al. (1986), the benefit of using asynchronous transfer for *in vitro* manipulated rabbit embryos was known prior to August 1995, and the skilled artisan at that time would have expected that *in vitro* manipulations of rabbit embryos, such as those used in nuclear transfer, would lead to a delay in the development of rabbit embryos that could be compensated by asynchronous embryo transfer. Thus, the successful cloning of rabbits, as reported by

Chesne, was accomplished using applicants' teachings together with a technique that was well known in the art to improve the efficiency of rabbit embryo development.

Horses

Third, the successful cloning of horses, as reported in Galli et al., also used procedures that were previously known to improve efficiencies of cloning prior to applicants' filing date.

Galli cites to Lazari et al., *J. Reprod. Fertil. Abstr. Ser.* 28, 73 (2002) for the proposition that both protein synthesis and protein phosphorylation must be inhibited. But Lazari et al. (Exhibit 3) merely describes activation studies done in horses with the known protein synthesis inhibitor cycloheximide (CHX) and known protein phosphorylation inhibitor DMAP, either alone or in combination. Lazari et al. report horse oocyte activation rates of 30.6% for CHX, 60% for DMAP, and 93% for CHX + DMAP.

CHX and DMAP were well known compounds used in ooctye activation protocols prior to applicants' filing date. These activation protocols included sequential protocols where cellular Ca+ levels were initially reduced (for example, by electrical pulse or ionomycin) and then maintained at low levels (for example, by a protein synthesis inhibitor (CHX) or a phosphorylation inhibitor (DMAP)). *See e.g.*, Susko-Parrish et al., *Dev Biol.* 1994 Dec;166(2):729-39 (Exhibit 4). Thus, Lazari et al. showed that conventional activation protocols, which existed as of applicants' filing date, could be successfully employed with horse oocytes.

Galli also references Lagutino et al., *Thieronology.* 59, 269 (2003) for evidence that the refinement of zona-free techniques aided their success. However, Lagutino et

al. (Exhibit 5) shows that zona-free manipulation is not critical for creating a horse embryo. Lagutino et al. compared the fusion rates of zona-intact and zona-free oocytes and subsequent cleavage rates of zona-intact and zona-free NT embryos. The observed rates were higher for zona-free, but zona-intact oocytes still had a 69% fusion rate and a 69% cleavage rate. Thus, Lagutino et al. confirms that conventional methods of fusion, which were available to one of skill in the art as of applicants' filing date were adequate to clone horses.

Rats

Fourth, Zhou reported successful cloning rats by using MG132, a protease inhibitor that reversibly blocks the first meiotic metaphase-anaphase transition. That is, the protease inhibitor MG132 helps maintain the oocytes in a **non-activated** state. This is just what the instant application teaches. The instant application specifically teaches reconstructing an embryo using a **non-activated** MII arrested ooctye. (Specification at 4, last paragraph.) Thus, Zhous' successful cloning of rats was performed according to applicants' teachings.

In summary, the Office's allegation that mice, rabbits, horses, and rats are not enabled by applicants' specification is in error. The Office's position does not take into account the general low efficiency of the cloning process. Moreover, while there may be techniques that will increase the efficiency of the cloning process, many of these techniques were known as of applicants' filing date. For each of these referenced species, applicants' teachings, either alone or in combination with well-known techniques, enable one of skill in the art to successfully clone the species. Accordingly, applicants respectfully request withdrawal of the rejection.

Claims 88-151 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly lacking an adequate written description. As claims 88-151 have been canceled, this rejection is moot.

Rejections under 35 U.S.C. § 102(b) and/or 103(a)

Claims 152-171 were rejected under 35 U.S.C. § 102(b) and/or 103(a) over several publications that teach embryo cloned cattle (Sims et al., 1993), sheep (McLaughlin et al., 1990), pigs (Prather et al., 1989), goats (Yong et al., 1991), mice (Cheong et al., 1993), and rabbits (Yang et al., 1992), and standardbred horses (Lawrence et al., 1993), and laboratory rats (Gonzales-Pacheco et al., 1993). It is the Office's position that the mammals of the cited prior art anticipate or make obvious applicants' claimed clones because there is no patentable distinction between the prior art mammals and applicants' claimed clones.

Applicants traverse the rejection. Applicants' claimed invention is neither anticipated by nor obvious over the cited prior art. Applicants will address anticipation and obviousness separately below.

Anticipation

For applicants' clone to be anticipated, the ***identical invention*** must be shown in as complete detail as contained in the patent claim. *See Richardson v. Suzuki Motor Co. Ltd.*, 9 U.S.P.Q.2d 1913,1920 (Fed. Cir. 1989). Anticipation is not shown by a **non-identical** prior art disclosure, even if it is "substantially the same" as the claimed invention. *Jamesbury Corp. v. Litton Indust. Prod., Inc.*, 225 U.S.P.Q. 253, 256 (Fed. Cir. 1985). Here, the cited prior art does not disclose the ***identical invention*** as applicants' invention.

Although the cited prior art does disclose certain animals, they are missing a limitation of applicants' claims, namely, one of the mammals is not a clone of a pre-existing, non-embryonic, donor mammal. For example, prior to applicants' invention, the only cloned mammals were made by "embryo cloning," and thus there was no *pre-existing, non-embryonic, donor mammal*, as recited in applicants' claims. Since the prior art clones and mammals are missing this limitation of applicant's claims, they cannot anticipate applicants' claimed invention. For the same reasons, applicants' clone is different than a naturally-occurring, sexually reproduced animal.

Moreover, although applicants' clone is a copy, or replica, of a previously known mammal, it is not ***identical*** to this donor mammal because, among other things, it occupies a different space and time than the previously known mammal. In addition, the two mammals will have phenotypic and behavioral differences. (February 26, 2003, Declaration of Dr. David Wells at ¶23.) Because of these differences, the prior art mammals cannot be ***identical*** to applicants' claimed clones. Since they are not ***identical***, the cited prior art mammals cannot anticipate applicants' claims, and applicants respectfully request withdrawal of the rejection.

Obviousness

The cited prior art cannot make obvious applicants' claims. Nowhere does the cited prior art teach or suggest that one of the mammals is a clone of a pre-existing, non-embryonic, donor mammal. Thus, the cited prior art cannot make applicants' claims obvious, and applicants respectfully request withdrawal of the rejection.

Moreover, applicants have shown that the claimed invention and the cited prior art are not identical or substantially identical. To the extent the Office may have made a

prima facie case under the decisions in Spada and In re Best, applicants have rebutted it by the evidence of record. (February 25, 2003, Declaration of David Wells).

Furthermore, a distinguishing feature of applicants' claimed mammals is that applicants' clone creates a situation that never existed prior to applicants' invention. This situation involves the existence of a non-embryonic, donor mammal prior to the existence of a clone of that mammal. Thus, one is able to physically examine applicants' donor mammal prior to the generation of a clone of that mammal. The benefits of this situation are readily apparent and cannot be considered obvious from the animals found in the prior art.

Prior to applicants' invention, no mammal existed that had obtained its entire set of chromosomes from a single parental mammal and, in this way, had the same set of chromosomes as its parent. By entire set of chromosomes applicants are referring to both of each pair of chromosomes. In contrast, all prior mammals obtained their set of chromosomes from two parents through sexual reproduction.

For example, the prior art mammals inherited one chromosome 1 and one chromosome 2 from each of its parents. In this way, both of its parents contributed to the prior art mammals' set of chromosomes. This is not the case with applicants' mammals. Applicants' clone inherits both sets of chromosomes from a single parent, namely, the "pre-existing, non-embryonic, donor mammal." Thus, applicants' clone inherits two copies of chromosome 1 and two copies of chromosome 2 from a single parent. This is an unexpected property of applicants' clone. This property of applicants' clone allows one to distinguish between it and sexually produced mammals. (February 26, 2003, Declaration of David Wells at ¶¶5-23.)

Conclusion

Applicants respectfully submit that this application is now in condition for allowance. If the Examiner believes that issues remain to be addressed before a Notice of Allowance, applicants respectfully request that the Examiner contact the undersigned to discuss any outstanding issues.

If there is any fee due in connection with the filing of this Amendment, please charge the fee to Deposit Account No. 06-0916.

Respectfully submitted,

Dated: November 16, 2005

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Factors Influencing the Results of Transfers of Rabbit Embryos Stored at −196° C

(dimethyl sulphoxide / culture / transfer to the recipients / implantation)

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Abstract. Rabbit embryos at the 8-cell and morula stages were frozen and stored at −196°C for 2−200 days. After thawing the embryos were examined for their viability in vitro and in vivo. In vitro, 62.5% of frozen 8-cell embryos and 81.4% of frozen morulae developed to blastocysts. In the control group of unfrozen embryos, 93.2% 8-cell embryos and 92.4% morulae developed to the blastocyst stage. Culture permitted a more reliable elimination of the embryos damaged during freezing and thawing. Embryos were transferred into the reproductive tracts of the recipients either directly after thawing or after 24 h in culture. Synchronous transfers of frozen rabbit embryos were not successful. After asynchronous transfers of morulae and blastocysts into the oviducts, implantation was 31.8% and 42.9%, respectively. After transfer of blastocysts into the uterine horns of the recipients, 47.6% embryos implanted.

Whittingham, Leibo and Mazur (1972) were the first to obtain viable young from embryos stored at −196°C upon transfer to foster mothers. With minor modifications, the method for mouse embryo storage has been used to store other mammalian embryos (Wilmut and Rowson 1973, Bank and Maurer 1974, Willadsen et al. 1974, Whittingham 1975, Bilton and Moore 1976). Only in mice were the results with embryos stored at −196°C comparable to those obtained with unfrozen embryos (Whittingham et al. 1977). In other mammalian species, the survival of frozen embryos is poor when compared with that of unfrozen embryos, particularly after transfer to foster mothers. Viability of frozen embryos after transfer is very low in rabbits. Only 10−15% of the embryos showed normal development after transfer (Bank and Maurer 1974, Whittingham and Adams 1976, Maurer and Haseman 1976). Better results were only exceptionally obtained (Tsunoda and Sugie 1977a). Why rabbit embryos stored at −196°C have low viability remains unknown.

In this study we have examined the factors which may markedly affect the results of transfers and which have not been investigated in detail previously: the selection of embryos for transfer and the effect of synchronization between donor and recipient.

Abbreviations: DMSO — dimethyl sulphoxide, HCG — human chorionic gonadotropin, FSH — follicle stimulating hormone, IU — international units.

MATERIAL AND METHODS

Recovery of embryos

Sexually mature female rabbits of various breeds were induced to superovulate by the subcutaneous injection of follicle stimulating hormone (Folicotropin, Spofa, Praha, Czechoslovakia) as described by Kennelly and Foote (1965). Twenty-four h after the last subcutaneous injection of FSH, the females were naturally mated and at the same time they received an intravenous injection of 100 IU of human chorionic gonadotropin (Preadyn, Spofa, Praha). Eight- to 16-cell embryos were obtained by flushing the oviducts 44 h after mating and HCG injection. Morulae (Plate XXI, Fig. 1/1) were flushed from the oviducts and uterine horns 68 h after mating and HCG. Only embryos of the desired type and with intact zona pellucida and mucin coat were used in further experiments. The medium used for flushing, freezing, culture and transfer was Krebs-Ringer's phosphate-buffered saline (pH 7.4) supplemented with lyophilized growth proteins of calf serum, 5 to 10 mg/ml (Institute of Sera and Vaccines, Praha), 5.5% isotonic glucose, 0.1 ml/ml streptomycin, 50 μg/ml and penicillin, 50 IU/ml.

Freezing and thawing of embryos

The method of Whittingham and Adams (1976) was modified as follows: After 5 min in medium with 0.5 M dimethyl sulphoxide and a further 5 min in 1.0 M DMSO, the embryos were transferred to vessels containing 0.1 to 0.3 ml of medium with 1.5 M DMSO. Embryos in groups of 10—15 were frozen in test tubes (1.5 × 8 cm) or plastic straws (0.5 ml) which were cut to 6 cm. After 10 min in medium with 1.5 M DMSO, the samples in test tubes and sealed plastic straws were transferred from room temperature directly to a cooling bath at −6°C. Two or three min later, the crystallization of the medium was induced by local cooling the walls of vessels. The rate of cooling was 1.0—1.3°C/min between −6 and −110°C. Freezing was performed in a ultracryostat N 180 (Prüfgeräte-Werk, Medingen, GDR). Upon reaching −110°C, the samples were transferred into liquid nitrogen and stored for 2—200 days.

The samples frozen to −196°C were thawed at rates of 7—9°C/min. Slow thawing was terminated at −5°C. The samples at −5°C were rapidly warmed to room temperature. The DMSO concentration was stepwise diluted by transfer of embryos into medium containing 0.7 M and 0.5 M DMSO, respectively, each time for 3—4 min. Embryos were kept in medium without DMSO for 10—30 min before further used.

The viability of embryos was checked by culture and by transfer into the recipients.

Culture of embryos

Embryos were cultured in drops of culture medium under paraffin oil (Brinster 1963) for 24—48 h at 37.5°C in an atmopshere of 5% CO_2 in air. Modified TCM 199 (Pavlok and McLaren 1972) was used as culture medium. For culture, the embryos after thawing were selected as follows: in experiment A/1 all embryos obtained from test tubes after thawing were used. In experiment A/2 undamaged embryos with an intact mucin coat and zona pellucida obtained from plastic straws were cultured. Unfrozen, normally developing embryos were cultured immediately after flushing from the genital tract.

At the end of the culture, the embryos were evaluated at a magnification of 100 to 300 and classified as "blastocysts", i.e., embryos with blastocoel occupying more than half of the embryo, with well visible embryoblast and without apparent damage to the trophoblast, and "degenerate and abnormal embryos", i.e.. all damaged and abnormally developing embryos.

Transfer of embryos

Ovulation in the recipients was induced by an intravenous injection of 50—100 IU of HCG. After laparotomy in the linea alba, the embryos in a small amount of the medium were transferred to the oviducts or uterine horns of the recipients. The number of embryos transferred into one recipient ranged between 5 and 11. Embryos were transferred either directly after thawing or after 24 h in culture. Some embryos were transferred into the recipients in which the length of the luteal phase of the cycle, as given by HCG administration, was synchronized with the embryo's age — "synchronous transfers". The other embryos were transferred into the recipients at a time when the embryos were 24 h older in comparison with the length of the luteal phase of the recipient's cycle — "asynchronous transfers". Individual experiments were arranged as follows: Synchronous transfers — Experiment B/1, 8-cell embryos (age 44 h) were transferred directly after thawing into the ampulla of the oviducts of the recipients used 44—48 h after HCG. Experiment B/2, 8-cell embryos cultured for 24 h were transferred into the uterine horns of the recipients 68—72 h after HCG. Asynchronous transfer — Experiment B/3, morulae (age 68 h) were transferred directly after thawing into the isthmus of the oviducts of the recipients 44—48 h after HCG. Experiment B/4, morulae cultured for 24 h were transferred into the isthmus of the oviducts of the recipients 68—72 h after HCG. Experiment B/5, morulae cultured for 24 h were transferred into the uterine horns of the recipients 68—72 h after HCG injection. Control was performed 12—14 days after the transfer. After laparotomy in the linea

Table 1. In vitro development of rabbit embryos frozen to −196°C and unfrozen embryos

Experiment	Deveop-mental stage	No. embryos frozen/No. embryos after thawing	No. cul-tured embryos	Length of culture (h)	Stage of embryos after culture degenerate and abnormal	blastocysts (%)*
A/1	8-cell	55/48	48	48	18	30 (62.5)
	morula	66/59	59	24	11	48 (81.4)
A/2	8-cell	60/60	30	48	13	17 (56.7)
	morula	86/86	72	24	19	53 (73.6)
Controls	8-cell	—	117	48	8	109 (93.2)
	morula	—	185	24	14	171 (92.4)

* Percentage was calculated from the number of cultured embryos.

alba, the number of implantations was determined. In 5 recipients a further control was made on day 25 after the transfer. Two recipients were allowed to go to term.

RESULTS

The development in vitro of rabbit embryos stored at −196°C and unfrozen embryos is summarized in Table 1. After thawing from test tubes (Exp. A/1), the proportion of 8-cell embryos and morulae developing to blastocysts was 62.5% and 81.4%. respectively. i.e., 54.5% and 72.7% of the total number of frozen embryos. Embryonic fragments and disintegrated coats indicated that embryos, which were lost during manipulation (12.7% 8-cell embryos and 10.6% morulae), had been damaged during freezing or thawing. Among unfrozen embryos, 93.2% 8-cell embryos and 92.4% morulae continued normal development.

Blastocysts derived from the frozen embryos after culture (Plate XXI, Fig. 1/4, 1/5) were comparable to those obtained from the unfrozen embryos (Plate XXI, Fig. 1/2, 1/3). Some embryos with damaged mucin coat (Plate XXI, Fig. 1/6), damaged investments and without investments continued development. Blastocysts developing from embryos with damaged investments were shedding the zona pellucida without preceding expansion. Some embryos with damaged investments rapidly degenerated.

Thirty (50%) 8-cell embryos and 14 (16%) morulae could not be cultured because of the damage to the zona pellucida and mucin coat (Plate XXII, Fig. 2/1—4), absence of investments (Plate XXII, Fig. 2/5), and damage to or loss of embryos (Plate XXII, Fig. 2/6—9). Of the morphologically "normal"

Table 2. Development of rabbit embryos frozen to −196°C after thawing and after transfer to foster mothers

Experiment	Developmental stage frozen	Developmental stage transferred	Recipient length of the luteal phase of the cycle (h)	Recipient site of transfer	No. recipients/No. embryos transferred	No. pregnant recipients/No. implantations (%)
B/1	8-cell	8-cell	48	oviduct**	6/56	—
B/2	8-cell	morula*	72	uterus	6/38	1/1 (2.6)
B/3	morula	morula	48	oviduct***	3/22	3/7 (31.8)
B/4	morula	blastocyst*	72	oviduct***	3/28	3/12 (42.9)
B/5	morula	blastocyst*	72	uterus	10/82	9/39 (47.6)

* Embryos cultured for 24 h.
** Transfer into the ampulla.
*** Transfer into the isthmus.

embryos selected for culture 56.7% 8-cell embryos and 73.6% morulae developed into blastocysts. Among frozen embryos, 28.3% 8-cell embryos and 61.6% morulae developed to the blastocyst stage (Exp. A/2). Among embryos selected in experiment A/2 for culture, 13 (43.8%) 8-cell embryos and 19 (26.4%) morulae showed retardation in development (Plate XXIII, Fig. 3/1, 3/2) did not develop (Plate XXIII, Fig. 3/3), only a few cells underwent division (Plate XXIII, Fig. 3/4) or degenerated (Plate XXIII, Fig. 3/5—9). The seemingly low effectiveness of the method in experiment A/2 was due to the elimination of undamaged embryos which had, however, investments damaged and gave generally poorer results during freezing in plastic ampoules. The methods of freezing and thawing used proved more advantageous for more advanced developmental stages (Exp. A/1, A/2).

Table 2 summarizes the results of transfers of rabbit embryos stored at −196°C. Synchronous transfers (Exp. B/1, B/2) were not successful. Of the 12 recipient animals, only one recipient became pregnant. Of the 94 transferred embryos, only one embryo implanted (Exp. B/2).

Asynchronous transfers (Exp. B/3, B/4) were more successful. After transfer of embryos into the oviducts of 6 recipients, all recipients became pregnant on days 12—14 after transfer. Of the 50 transferred embryos, 19 implantations, 31.8% morulae and 42.9% blastocysts (Exp. B/3, B/4) were observed. The best results were obtained with asynchronous transfers of blastocysts into the uterine horns. Of the 10 recipients, 9 became pregnant and of the 82 transferred embryos, 39 (47.6%) implantations were seen, i.e., 52% embryos developed from blastocysts transferred to pregnant recipients (Exp. 5/5).

Normal development of embryos observed on day 25 after transfer in 5 recipients and live young born to 2 recipients confirmed that embryos stored at −196°C are capable of normal development.

DISCUSSION

In our experiments the viability of rabbit embryos frozen to −196°C and thawed was comparable to the results obtained by Bank and Maurer (1974), Whittingham and Adams (1976), Maurer and Haseman (1976) and Tsunoda and Sugie (1977b). The results probably reflect the present possibilities of the method based on slow freezing to temperatures lower than −60°C and on slow thawing. The lower viability of embryos frozen in plastic straws could have been due to some unfavourable properties of plastic material as was also the case in the experiments of Whittingham et al. (1977).

The development in vivo depends on the selection of embryos. High mortality after transfer of damaged and abnormal embryos (Shea et al. 1976, Bilton and Moore 1977, Polge and Willadsen 1978) and the degeneration of damaged embryos during culture, as observed in our experiments, have demonstrated that such embryos are not convenient for transfer. Selection of rabbit embryos according to the morphological condition need not be reliable. We showed that some of the morphologically "intact" embryos degenerated or developed abnormally already after short-term culture. The short-term culture of embryos after freezing proved to be a convenient method for an additional elimination of embryos which had been apparently damaged at the subcellular level during freezing and thawing and it appeared to be suitable for the selection of embryos capable of normal development. If suitable culture systems are used, short-term culture has no adverse effect on further development of embryos (Adams 1970).

In selecting embryos for transfers we also paid attention to the condition of the non-cellular investments of rabbit embryos, the zona pellucida and the mucin coat. Mechanical damage to the investments increases the risks of damage during further manipulation in vitro and often causes degeneration and loss of embryos. In some cases, the embryos continued development, even in damaged investments, and deaths occurred at a later time as a result of their premature shedding the investments. A marked decrease in implantation already after the reduction of the mucin coat has been reported by Greenwald (1962), the necessity of expansion of the blastocyst for the initiation of implantation by Chang (1950) and the effect of the mucin coat on the formation of the rabbit preimplantation blastocyst by Kane (1975). Thus, they have indirectly demonstrated that embryos with damaged investments must be discarded.

All the factors involved in the damage to the investments of rabbit embryos are not yet known. In our experiments we failed to confirm the results

of Whittingham and Adams (1976), who decreased the incidence of embryos with damaged investments from 29% to merely 9% by prolonging slow freezing from −80°C to −110°C. The effectiveness of long-term preservation of rabbit embryos will be increased if the causes of the damage to the investments are known.

Synchronization between donors and recipients is of primary importance for the results of transfer. We have shown that currently used synchronous transfers (Chang and Pickworth 1969, Rowson et al. 1969) are not suitable for rabbit embryos stored at −196°C. This may be due to the retardation in development caused by the sojourn in unfavourable conditions as observed both in frozen embryos (Whittingham 1977) and in embryos stored at 10°C (Anderson and Foote 1975) or in cultured embryos (Bowman and McLaren 1970). During synchronous transfers so affected embryos are placed in an environment which does not correspond to their developmental capacity. The site of transfer also plays a role. The uterine environment proved not suitable, mainly for the early developmental stages (Chang 1950, Adams 1970). Actually the transfer of morulae into the uterine horns of the synchronized recipients may have caused the failure or a low level of implantation not only in our experiments but also in those of Bank and Maurer (1974), Maurer and Haseman (1976) and Whittingham and Adams (1976). The transfer of later developmental stages into the oviducts may favourably affect further development of the embryos (Tsunoda and Sugie 1977a). The results of transfers were markedly improved by asynchronous transfers. More than 50% of the implanting embryos corresponded to the results obtained with unfrozen embryos (Adams 1962, Binkerd and Anderson 1979). It appeared that rabbit embryos, like mouse embryos (Whittingham and Anderson 1976), required a certain time for the restoration of the metabolism and for the desired level of synchronization which was disturbed by storage at very low temperatures.

The high efficacy of frozen embryo transfers suggests that rabbit embryos might be more widely used in various areas of reproduction biology. Some questions, for example, the variations in the number of embryos implanting in individual recipients (10—80%), cannot be answered unless further studies are undertaken. In this regard, factors other than low temperatures are involved, because similar variations are also observed with unfrozen embryos.

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В. Ланда

Факторы, влияющие на результаты пересадки кроличьих эмбрионов, хранившихся при —196 С

Резюме. Кроличьи эмбрионы в стадии восьми клеток и морул замораживали при температуре —196 °С и хранили 2—200 суток. После оттаивания определяли жизнеспособность эмбрионов в условиях in vitro и in vivo. В течение культивации in vitro стадии бластоцист достигали до 62,5% замороженных 8-клеточных эмбрионов и 81,4% замороженных морул. Из контрольной группы без замораживания продолжали свое развитие 93,2% 8-клеточных эмбрионов и 92,4% морул. Культивация in vitro позволила более надежно исключать эмбрионы, поврежденные в течение замораживания и оттаивания. В половой тракт реципиенток эмбрионы переносили или немедленно после оттаивания, или после 24-часовой культивации in vitro. Синхронный перенос замороженных кроличьих эмбрионов не давал успеха. После несинхронного переноса морул и бластоцист в яйцевод имплантировались соответственно 31,8% и 42,9% эмбрионов. После переноса бластоцист в углы матки реципиенток имплантировались 47,6% эмбрионов.

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In vitro fertilization and embryo transfer of pre-ovulatory rabbit oocytes

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Summary

The objective of this study was to develop an effective and successful technique of in vitro fertilization in rabbits that could lead us to start treatment of infertile couples by using extracorporal fertilization.

Pre-ovulatory oocytes were harvested from follicles of virgin does 9 h after induction of ovulation by hCG injection, and incubated with in vivo capacitated sperm for 5 h. An 82% in vitro fertilization rate was achieved. The fertilized ova were cultivated for an additional 20, 44 and 68 h, and until they reached the hatched blastocyst stage. Most of these embryos (439) were transferred to 42 pseudopregnant recipients, asynchronous in the sense that they had been given hCG injections 0, 6, 12, 18, 24, 36 and 42 h later than the rabbits from which the ova had been obtained. With this type of synchronicity implantation rates of 31, 78, 52, 33, 52, 33, 51 and 10% were achieved, respectively.

One hundred and twenty-eight embryos were not transferred to recipients but cultured in Ham's F-10 medium supplemented with 20% FCS. 77% of these embryos reached the stage of hatched blastocyst. A total of 143 young were born after 28–32 days of gestation and all are fertile till the third generation.

Our results were obtained in a consecutive series of experiments, and demonstrated that in vitro fertilization can be made to work quite well in the rabbit, which in the early stages of embryogenesis has sufficient similarity to the human to make it a useful model for those wishing to acquire techniques applicable to human fertilization in vitro and embryo transfer.

in vitro fertilization; embryo transfer; synchronization; rabbit; oocytes

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Introduction

Nineteen eighty-one was the year of the final breakthrough in the field of in vitro fertilization as a method of treating infertile women. About 600 children conceived in this way were born throughout the world [1–3]. Despite these successes, however, we cannot yet be satisfied with the maximum pregnancy rate presently achievable after in vitro fertilization and embryo transfer. One of the problems that still have to be solved is the 'desynchronization' between the embryo and endocrinal status in the woman – a particularly important point in human subjects where donor and recipient are identical. While, after follicle puncture, the luteal phase develops at a normal rate [4], the in vitro conditions obtaining during the cultivation of the embryo give rise to a delay in the growth of the extracorporal embryos.

The objective of this study was to investigate the extent of the 'desynchronization' for varying cultivation times, in animal experiments. For various reasons, which we have already discussed elsewhere [5], we selected the rabbit as our experimental animal. We have been working on the in vitro fertilization technique intensively for a number of years now, and have developed a reproducibly functioning in vitro fertilization model for pre-ovulatory rabbit oocytes [6]. A number of investigators [7–9] have reported on the influences of synchronicity, i.e. between the age of the transferred embryos and the endocrinal status (time elapsed after ovulation) of the recipient, on the results of embryo transfer in various laboratory animals.

Our experiments were designed to enable us to transfer pre-ovulatory rabbit oocytes, fertilized in vitro and cultured for periods varying between 24 and 72 h, to synchronous and asynchronous foster animals. In the latter animals, ovulation was induced after a varying delay vis-a-vis the oocyte donors.

Material and methods

Animals

Cross-bred does were used for the collection of follicular oocytes, the recovery of capacitated uterine sperm, and as recipients. All animals were at least 6 months old and virgins. They were caged individually for no less than 3 weeks before being used, to avoid pseudopregnancy. Males and females were housed in separate air-conditioned (21°C) rooms. Lights were on in the rooms for 12 h during each 24-h period. Animals were fed rabbit chow and provided with water ad libitum.

Media and culture conditions

Brackett's defined medium [10] supplemented with 3 mg/ml bovine serum albumin (BSA) was used for in vitro fertilization and recovery of capacitated sperm. Ham's F-10 supplemented with 20% inactivated (30 min at 56°C) fetal calf serum (FCS) served as culture medium. Media were sterilized by positive-pressure filtration (0.22 μm millipore filters) and equilibrated with a gas phase of 5% CO_2, 5% O_2 and 90% N_2. The same gas mixture was used during incubation in an anaerobic jar inside an incubator at 37°C. To achieve a high relative humidity, the gas was bubbled through distilled water at the bottom of the anaerobic jar.

Oocyte donors

The donor females were superovulated by subcutaneous injections of 0.4 mg FSH (Burns Biotec Laboratories incorporated Omaha, Nebraska) in 4 single doses over 3 days. Ovulation was induced by an intravenous injection of 100 I.U. of hCG (Primogonyl, Schering AG, Berlin-Bergkamen) given together with the last priming dose of FSH. Ovum donors were killed 9 h later. The ovaries were excised, submerged in Brackett's medium and kept in petri dishes at 37°C. After puncturing all follicles seen over the surface of the ovaries, the oocytes were aspirated with a finely pointed glass pipette. Follicular ova in surrounding cumulus cells were washed twice in Brackett's medium and kept under described conditions in the incubator until the recovery of capacitated sperm.

Collection of capacitated spermatozoa

The ejaculates of 6–8 bucks were collected by an artificial vagina using a teaser doe. To produce capacitated spermatozoa, does were inseminated intravaginally with 4 ml of pooled semen. Just after the insemination the animals received an injection of 100 I.U. of hCG intravenously to induce ovulation. Capacitator does were laparotomized 14 h after insemination [11,12]. Both uteri were flushed with 2–3 ml of warm Brackett's medium after puncturing the uterus near the utero-tubal junction with a blunt needle. To prevent leakage of the flushing medium the cervix was clamped with an artery forceps. The fluid containing capacitated sperm was collected in petri dishes, and covered with autoclaved paraffin oil. The petri dishes were wrapped in aluminium foil to prevent direct exposure to light [13]. Then the oviducts were flushed to collect the fertilized eggs, which were cultivated in Ham's F-10 and used as controls.

Handling of the gametes

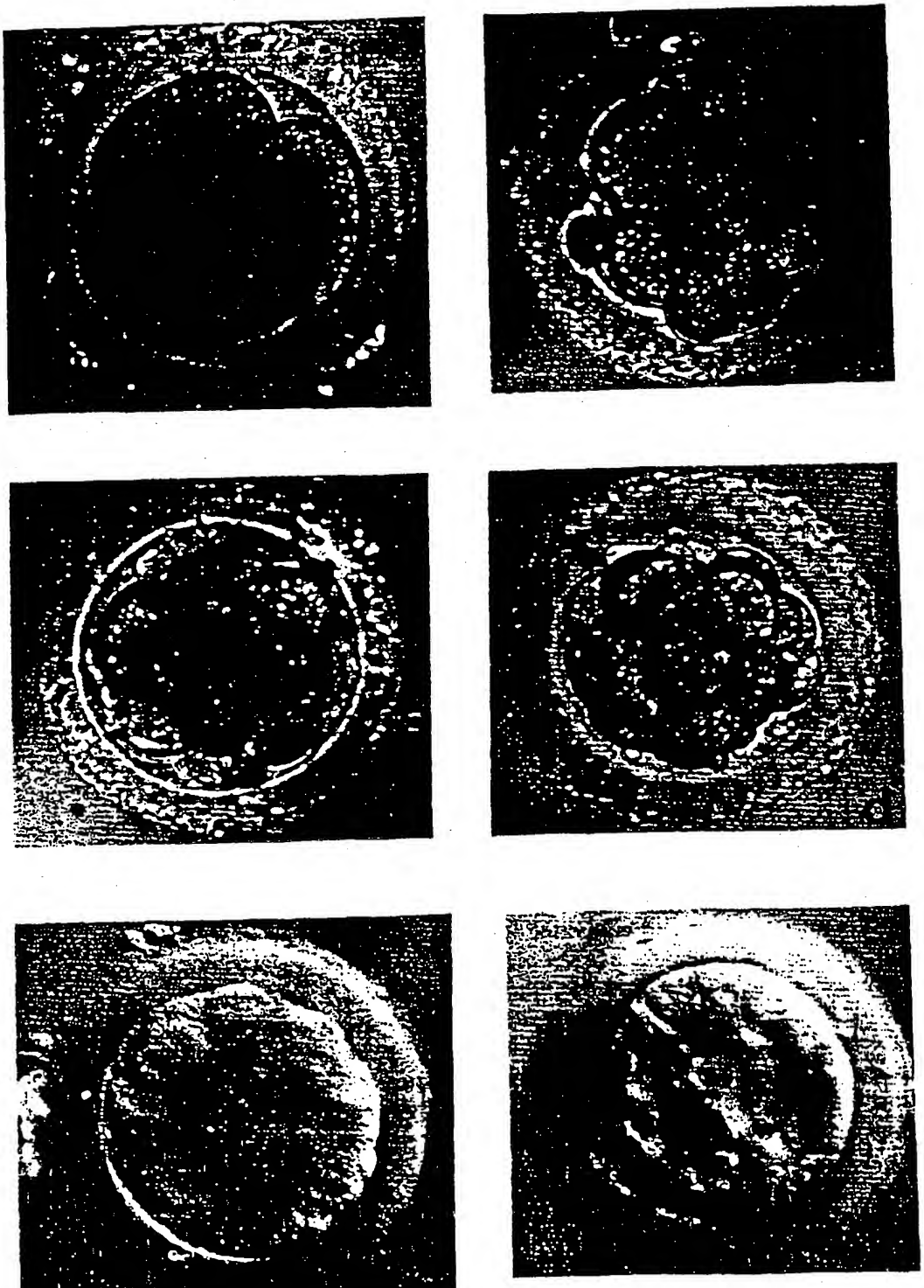
Ten to thirty follicular oocytes were added to one petri dish containing 2–3 ml capacitated sperm flushed from one side. Details of our technique have been described in a recent publication [5]. The incubation of the gametes was carried out under described conditions. Five hours later the ova were washed twice in Ham's F-10 culture medium and cultivated for a further 20–72 h.

Criterion of successful in vitro fertilization

Normal cleavage to the 2- or 4-cell stage was used as the sole criterion of successful fertilization. A second polar body, sperm penetration of the zona pellucida and unequal blastomeres were not considered as criteria of successful fertilization. Adhering granulosa cells only occasionally needed to be removed mechanically to permit the blastomeres to be seen clearly on microscopy.

Embryo transfer

The recipients were made pseudopregnant by an intravenous injection of 100 I.U. hCG given 0, 6, 12, 18, 24, 30, 36, and 42 h after the oocyte donors received their ovulatory injection of hCG. These intervals were intended to compensate for the delayed development of the embryos due to in vitro conditions. Preimplantation embryos from the 4-cell stage to early blastocytes (Fig. 1a–f), which had been



cultured for 24–72 h following in vitro fertilization, were transferred with the help of a blunt capillary glass pipette into the oviducts of the foster mothers through flank incisions.

Control of successful embryo transfer

Recipients were laparotomized after 15 days gestation in order to count the number of implantations and to distinguish between normal and retarded, apparently resorbing, fetuses. All pregnant does were allowed to deliver their offspring.

Results

Collected eggs

The number of follicles were counted by inspection of the excised ovaries. A total of 1353 (95%) ova were harvested from 1423 follicles in 82 ovaries obtained from 41 does. Among these, 166 oocytes from atretic follicles were discarded. Red-colored, unruptured follicles (folliculum haemorrhagicum) were neither counted nor aspirated. During our experiments not a single ovulation had occurred 9 h after hCG injection.

In vitro fertilization rate

An 82% in vitro fertilization rate of preovulatory oocytes recovered 9 h after hCG injection (Table I) was achieved. Owing to a lack of recipients, 167 fertilized ova could not be used for transfer experiments. These were cultured for an additional 4–5 days and 128 (77%) blastocysts developed.

Results of embryo transfer

Table II(a) contains data on the transfer of embryos, cultured for 24–26 h, to the oviducts of synchronous pseudopregnant recipients that had received their hCG injection at the same time as the donor animals. Sixteen (31%) implantations were achieved among 52 transferred embryos; only 2 resorptions occurred. Fourteen viable young were born on the 31st day of pregnancy.

The transfer results for embryos cultured for 24–26 h to recipients that had received their hCG injections 6 h later than the ovum donor are summarized in Table II(b). On the 15th day of pregnancy 54 (78%) implantations had taken place (after 69 embryos had been transferred). Among these 54 implantations 7 resorptions were seen. A total of 47 viable young were born at term.

Table II(c) contains data on the transfer of embryos cultured for 24–26 h to foster mothers that had received their hCG injection 12 h later than the donor animals. On the 15th day of pregnancy 30 (45%) fetuses had developed from 67 transferred embryos, and an additional 5 resorptions were counted. Twenty-seven viable young were born at term.

Fig. 1. In vitro fertilized embryos. (a) 4-cell-stage embryo 24 h after in vitro fertilization (i.v.f.). (b) 8-cell-stage embryo 26 h after i.v.f. (c) 16-cell-stage embryo 48 h after i.v.f. (d) A morula-stage embryo 48 h after i.v.f. (e) Late morula-stage embryo 72 h after i.v.f. (f) Early blastocyst 72 h after i.v.f.

TABLE I

In vitro fertilization results of preovulatory rabbit oocytes

Expt. No.	No. of animals	No. of collected oocytes	Fertilized oocytes 2–4- or 8-cell stage		No. of embryos cultured 4–5 days	Blastocysts	
			No.	%		No.	%
28	41	1187 *	972	82	167	128	77

* Total number of oocytes 1353; 166 oocytes of atretic follicles were discarded.

The results obtained with embryos cultured for 48 h and transferred to the oviducts of recipients that had received their hCG injection 18 h later than the oocyte donor are summarized in Table III(a). During a monitoring laparotomy on the 15th day of pregnancy, 16 (26%) fetuses had developed from 61 transferred embryos, and 4 resorptions were counted. Ten viable young were born at term.

Table III(b) shows data on the transfer of embryos cultured for 48 h, to the oviducts of pseudopregnant foster mothers that had received their hCG injection 34 h earlier. Thirty-two (52%) implantations were achieved among 61 transferred embryos; 13 resorptions occurred. Nineteen viable young were delivered on the 31st to 32nd day of gestation.

Table IV(a) contains data on the transfer of embryos cultured for 72 h to the oviducts of pseudopregnant recipients that had received their hCG injections 52 h earlier. Fifteen (33%) implantations were achieved among 46 transferred embryos. A total of 10 viable young were born on the 31st to 32nd day of gestation, and 4 resorptions occurred.

Table IV(b) shows the results of the transfer of embryos cultured for 72 h to the oviducts of pseudopregnant foster mothers that had received hCG injection 46 h earlier. Twenty-two (51%) implantations were achieved among 43 transferred embryos, and 5 resorptions occurred. At the end of the gestation period, 14 viable young were born.

TABLE II

Transfer results of in vitro fertilized rabbit oocytes cultured for 24–26 h

Recipients were given hCG (a) at the same time as, (b) 6 h and (c) 12 h later than the rabbits from whom the ova were obtained.

	No. of animals	No. of embryos transferred	No. of implantations at day 15		Fetuses		Resorption		No. of young born
			No.	%	No.	%	No.	%	
(a)	6	52	16	31	14	27	2	4	14
(b)	6	69	54	78	47	68	7	10	47
(c)	6	67	35	52	30	45	5	7	27

Table IV(c) summarizes the results obtained with embryos cultured for 72 h and transferred to the oviducts of pseudopregnant recipients that had received their hCG injection 40 h before the transfer. On the 15th day of gestation 2 (5%) fetuses had developed among 40 transferred embryos, and 2 resorptions had occurred. Only 2 viable young were born at the end of the gestation period.

Discussion

In earlier reports, we showed that the in vitro fertilization of pre-ovulatory rabbit oocytes could be effected at our laboratory in a reproducible and reliable manner [5,6]. The results presented here with considerably greater numbers of animals confirm those obtained earlier. In summary, our relative and absolute recovery rate for oocytes, and the in vitro fertilization rates, exceed those obtained earlier, irrespective of whether the various investigators made use of preovulatory or already ovulated tubal oocytes [9,10,12,14–17]. However, this was merely an interesting 'by-product' of our investigation.

The actual purpose of this study was to determine the loss of synchronicity of rabbit oocytes fertilized in vitro, for varying times of cultivation. The answer to this

TABLE III

Transfer results of in vitro fertilized rabbit oocytes cultured for 48 h

Recipients were given hCG 18 h (a) and 24 h (b) later than the rabbit from whom ova were obtained.

	No. of Animals	No. of embryos transferred	No. of implantations at day 15		Fetuses		Resorption		No. of young born
			No.	%	No.	%	No.	%	
(a)	6	61	20	33	16	26	4	7	10
(b)	6	61	32	52	19	31	13	21	19

TABLE IV

Transfer results of in vitro fertilized rabbit oocytes cultured for 72 h

Recipients were given hCG 30 h (a), 36 h (b) and 42 h (c) later than the rabbits from whom ova were obtained.

	No. of animals	No. of embryos transferred	No. of implantations at day 15		Fetuses		Resorption		No. of young born
			No.	%	No.	%	No.	%	
(a)	4	46	15	33	11	24	4	9	10
(b)	4	43	22	51	17	40	5	12	14
(c)	4	40	4	10	2	5	2	5	2

question is of importance, since the loss of synchronicity following in vitro fertilization and embryo transfer is probably of considerable significance in the human situation. The animal model 'rabbit' is particularly suitable for synchronicity studies, since the time of ovulation of this reflex ovulator can be determined exactly by the injection of hormones.

Our transfer results seem to prove that the in vitro conditions apparently lead to a delay in the development of the embryos. This loss of synchronicity can be, in part, compensated by using 'asynchronous' recipient animals, that is, the foster animals are induced to ovulate somewhat later than the oocyte donors. The longer the in vitro fertilized embryos are cultivated, the greater is the loss of synchronicity. In our system of in vitro fertilization with subsequent embryo transfer, the following asynchronicities of the recipient animals proved optimal:

Cultivation duration after IVF	Optimal asynchronicity of the recipients
24 h	minus 6 h
48 h	minus 24 h
72 h	minus 36 h

Even when full advantage is taken of the optimal asynchronicities of the recipients, the transfer successes, determined by the number of implantations and viable progeny, decrease with increasing culture time.

The last two facts show that our cultivation conditions are not yet optimal. Here, we believe, is a large area for future research. We expect similar laws to apply in in vitro fertilization in human subjects, too.

Steptoe and Edwards [18] achieved their first successes only after reducing their cultivation time. The overall very modest success obtained with embryo transfer in humans is attributed, among other things, to the loss of synchronicity, which still pertains, despite a reduction in culture time.

Using our in vitro fertilization model in the rabbit, we were able to produce 143 living progeny. This is the largest number so far reported in a consecutive series in this species.

A particularly noteworthy observation is, we believe, the fact that, despite very thorough examinations and subsequent observation, we were unable to find any malformations in the progeny. These animals produced by in vitro fertilization have already reproduced in the third generation. We are using them in our 'colony' for further in vitro fertilization experiments. There is no evidence to suggest that their fertility is in any way impaired.

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daily for thirty two days during winter months of January and February. Estrus was detected by visual symptoms, parading a vasectomized bull twice daily and confirmed by monitoring plasma progesterone concentrations. Progesterone concentrations were determined by a simple, direct RIA. The sensitivity of assay was 8 pg/tube. The intra- and inter-assay coefficients of variation were 13.4 and 16.9 percent, respectively. Plasma progesterone concentrations increased from 0.40 ± 0.02 and 0.47 ± 0.03 ng/ml during periestrus phase to 0.74 ± 0.10 and 0.94 ± 0.08 ng/ml during early luteal phase and then further ($P<0.05$) to 1.94 ± 0.22 and 1.39 ± 0.13 ng/ml during midluteal phase following which declined ($P<0.05$) to 0.63 ± 0.16 and 0.95 ± 0.19 ng/ml during late luteal phase in cows exhibited overt estrus and silent estrus, respectively. Plasma progesterone concentrations increased from 0.42 ± 0.02 and 0.38 ± 0.02 ng/ml during periestrus phase to 0.66 ± 0.12 and 0.51 ± 0.07 ng/ml during early luteal phase and then further ($P<0.01$) to 1.55 ± 0.33 and 1.30 ± 0.13 ng/ml during midluteal phase following which declined ($P<0.01$) to 1.18 ± 0.27 and 0.66 ± 0.13 ng/ml during late luteal phase in buffaloes exhibited overt estrus and silent estrus, respectively. Overall plasma progesterone levels were lower in cows and buffaloes that exhibited silent estrus compared to overt estrus and might be responsible for poor expression of estrus.

73. Synergic effect of cycloexemide and 6-DMAP on activation of equine and bovine oocytes.

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Cycloheximide (CHX) and 6-DMAP at concentration of 10μg/ml and 2mM respectively are used routinely for the activation of bovine oocytes in association with ionomycin. By contrast, little information is available regarding their effect on the equine oocyte and also their use in combination. For this study oocytes were matured *in vitro* for 24h (bovine) or 28h (equine) in medium TCM 199 supplemented with 10% FCS (bovine) or 10% Serum Replacement (equine, Life Technologies) and 0.1 IU of LH and FSH. Matured oocytes were activated with 5 μM ionomycin for 4 minutes, rinsed and transferred in medium SOF-AA-BSA supplemented with CHX or DMAP or for 4h. Then they were rinsed twice and transferred in medium without inhibitors and 10 to 14 h later they were fixed and stained with laomoid. All experiments were done in three replicates. For the equine oocytes the activation rate was 30.6% (11/36) for CHX (10μg/ml), 60% (15/25) for DMAP (2mM) and 93.1% (27/29) for CHX + DMAP indicating a clear synergic effect between the compounds (chi square test, $p<0.05$). For the bovine oocytes we tested also lower concentrations. When the compounds were used alone we observed a progressive reduction of the activation rate: for CHX 69% (30/44, 10 μg/ml), 47% (21/45, 5 μg/ml), 46% (19/41, 2.5 μg/ml) and for DMAP 100% (30/30, 2mM), 93% (26/28, 1mM), 43% (13/30, 0.5mM). However when the inhibitors were used together the activation rate was 100% at the two combinations tested: 100% (31/31, 5μg/ml CHX + 1mM DMAP) and 100% (35/35, 2.5μg/ml CHX + 0.5mM DMAP). Comparing these data with those obtained with the inhibitors used separately at 2.5μg/ml for CHX and 0.5mM for DMAP we found a significant increase in activation rate (46% and 43% *versus* 100%, chi square test, $p<0.05$). This study demonstrates that CXH and DMAP act in a synergic manner to induce oocyte activation both in equine and in bovine.

74. Effect of *in vitro* maturation on the expression of the peroxiredoxin family of antioxidant enzymes in bovine oocyte-cumulus complexes.

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The expression of peroxiredoxins (PRDX1 to 6), a recently characterized family of antioxidant enzymes, has been studied in bovine oocyte-cumulus complexes (COCs) before and after *in vitro* maturation by multiplex RT-PCR.

Pools of COCs, denuded oocytes and cumulus cells have been frozen at –80°C. Total RNA was extracted in presence of glycogen (2%). The reverse transcription was done using Superscript II (Life Technologies), while Ex Taq (TaKaRa) was used for the multiplex and standard polymerase chain reactions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control.

The six peroxiredoxins were detected by multiplex RT-PCR both in COCs and denuded oocytes before and after maturation. In cumulus cells, all peroxiredoxins were also expressed before and after maturation, except PRDX6 that was not detectable by multiplex RT-PCR before maturation. Standard PCR confirmed an important overexpression of PRDX6 in cumulus cells after maturation.

Our results show that most peroxiredoxins are expressed in bovine cumulus cells and oocytes. Furthermore, transcription of PRDX6 is induced in cumulus cells during maturation. PRDX6 is the only peroxiredoxin with phospholipase A2 activity, which contributes with cyclo-oxygenase to the synthesis of prostaglandins. Since prostanoids are a major factor involved in cumulus expansion, PRDX6 could play an important role in COC maturation.

G Leyens is a Research Fellow of the Fonds National de la Recherche Scientifique de Belgique.

75. Effect of ovarian status on maturation potential of sheep oocytes.

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Number of viable embryos in superovulated ewes are affected by presence of large follicles (≥6mm in size) and/or Corpus luteum at start gonadotrophin treatments (Gonzalez-Bulnes *et al.* 1999, JRF Abstracts 24: 6; Gonzalez-Bulnes *et al.* 2000, 2000 ESDAR Newsletter: 14), suggesting that a proportion of the smaller follicles stimulated to grow and ovulate by exogenous FSH may be in early stages of atresia and subsequently developmental competence of its oocytes should be compromised. This study have tested this hypothesis by assessing possible effects from ovarian status at start FSH treatment on the ability of oocytes from smaller follicles to undergo nuclear maturation *in vitro*. A

1: Dev Biol. 1994 Dec;166(2):729-39.

ELSEVIER FULL-TEXT ARTICLE

Inhibition of protein kinases after an induced calcium transient causes transition of bovine oocytes to embryonic cycles without meiotic completion.

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We have examined the response of bovine oocytes matured in vitro for 24 hr to parthenogenic activation using compounds that increase intracellular calcium (ionomycin) or inhibit protein phosphorylation (6-dimethylaminopurine, DMAP). Treatment with ionomycin alone caused resumption of meiosis (57.8 +/- 7.8%) but not pronuclear formation (8.9 +/- 7.3%). DMAP alone did not cause resumption of meiosis or pronuclear formation. Sequential treatment with ionomycin (5 microM for 4 min) immediately followed by DMAP (1.9 mM for 5 hr) resulted in activation that led to pronuclear formation (80.5 +/- 13.1%). Completion of meiosis, however, was bypassed as evidenced by only one polar body and one pronucleus present in activated parthenogenones. It was necessary to incubate the oocytes for at least 3 hr in DMAP to obtain high rates of activation (76.6 +/- 9.8%) and development to blastocysts (21.1 +/- 1.5%). Temporal separation of the two treatments resulted in a decrease in oocytes with one pronucleus and one polar body (uniformly diploid parthenogenones) and an increase in a mixture of diploid and haploid parthenogenones since DMAP was capable of causing transition to interphase of all chromatin configurations after anaphase commenced and prior to metaphase arrest. Parthenotes produced with ionomycin and DMAP that developed to the blastocyst stage had high cell numbers (70 to 88 cells) and were able to cause extended cycles in 33.3% of recipient cattle after nonsurgical transfer to the uterus. Response of the bovine oocyte arrested in metaphase II to different activation stimuli was also found to show age-dependent changes in pattern of activation response and developmental competence.

NUCLEAR TRANSFER IN HORSES

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In this study we investigated the developmental ability of horse embryos after nuclear transfer (NT) with cumulus cells or adult skin fibroblasts. Equine oocytes with compact and expanded cumulus were matured for 20–22 h in TCM199 with 10% FCS, 0.1 IU/ml LH and 0.1 IU/ml FSH, decumulated and enucleated. Cumulus cells for NT were obtained by trypsinisation from maturing oocytes with compact cumulus. Fibroblasts of passage 1–6 were cultured in TCM199/DMEM with 10% FCS to establish a confluent monolayer or were serum starved for 1–4 days. NT-embryos were obtained: (1) by transfer of a cell under the zona pellucida and fusion in 0.3 M mannitol by double pulse of 30 μs DC 2.4 kV/cm; or (2) by attachment of zona pellucida free (0.5% pronase) oocytes to a single cell in TCM199 with 200 μg/ml of lectin (PHAP) and fusion by double pulse of 30 μs DC 1.2 kV/cm. The fusion rate of zona-intact ooplasts with cumulus cells was 69%, while 100% of zona-free ooplasts fused with cumulus cells and 97% with fibroblasts ($P \leq 0.05$). One 2 h post fusion embryos were activated by 5 μM ionomycin for 4 min and incubated in the mixture of 5 μg/ml cycloheximide and 1 mM 6-DMAP in SOFaa for 4 h. Embryos were cultured in SOFaa in 5% CO_2, 5% O_2 at 38.5 °C. The data were compared by Chi-square test. The cleavage rate of zona-free NT-embryos (84–88%) did not depend on quality of oocyte cumulus or origin of nuclei and was significantly higher than of zona-intact NT-embryos with cumulus cell nuclei (69.2%, $P \leq 0.05$). However, this difference could be due to technical difficulties. Further work is needed to confirm it. There were 1.3–1.9% of NT-embryos with cumulus cell nuclei and 3.8–4.4% of NT-embryos with fibroblast nuclei that formed blastocysts on Day 8. This study shows that the development of NT-embryos to the blastocyst stage is not affected by the morphology of the oocyte cumulus before maturation and by the presence/absence of zona pellucida.

Nuclear donor	Cumulus morphology	Zona pellucida	Number of fused embryos	Cleaved (%)	Blastocysts (%)
Cumulus cells	Compact	+	78	54 (69.2)[a]	1 (1.3)
	Expanded	–	104	88 (84.6)[b]	2 (1.9)
Adult fibroblasts	Compact	–	205	180 (87.8)[b]	9 (4.4)
	Expanded	–	52	45 (86.5)[b]	2 (3.8)

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